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14. ABSTRACT

Uncontrolled proliferation of tumor cells due to failure of immune-surveillance has been linked to cancer development. Regulatory T cells (T_{reg}) play a critical role in immune tolerance by suppressing immune responses to the body's own antigens. The tyrosine phosphatase SHP-1 is a well-known negative regulator of T cell signaling that also affects the generation of T_{reg} cells. Interestingly, mice with decreased levels of SHP-1 protein show a high occurrence of breast cancer. The objective of the studies proposed in the concept award is to test the hypothesis that the CD4+CD25+ regulatory T cell population plays a role in the increased incidence of breast tumors we have observed in *me*/+ mice. During the first year of the award, we have characterized transgenic mice expressing a dominant negative mutant of SHP-1 in the T cell lineage. Analyses of these mice have demonstrated a T cell autonomous effect of SHP-1 as assessed by TCR/CD3-mediated hyper-proliferation of the mice expressing the dominant negative mutant compared to non-expressers. Moreover in preliminary data, it was observed that expressers show an increase in CD4+CD25+ Treg cells compared to non-expressers making these transgenic mice a powerful model system to directly test the hypothesis that SHP-1 deficiency promotes the development/onset of breast cancer by increasing the number of regulatory T cells.

15. SUBJECT TERMS

regulatory T cells, protein tyrosine phosphatase, tumor immunology

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Table of Contents

Final Report (Years 1 and 2) for Concept Award
"Role of the Tyrosine Phosphatase SHP-1 and Regulatory T cells in Breast Cancer"

Introduction

The failure of immune-surveillance by the body allowing uncontrolled proliferation of tumor cells has been linked to cancer development. Regulatory T cells (T_{reg}) play a critical role in immune tolerance to self by suppressing immune responses to autoantigens. For a variety of cancers, including breast cancer, increased T_{reg} cell numbers have been reported. The tyrosine phosphatase SHP-1 is a well-recognized negative regulator of T cell signaling that also affects the generation of T_{reg} cells (1). Interestingly, mice heterozygous for the *motheaten* (*me*) allele, which express decreased SHP-1 protein levels, show a high occurrence of breast cancer.
The objective of the studies proposed in the concept award is to test the hypothesis that the CD4+CD25+ regulatory T cell population plays a role in the increased incidence of breast tumors we have observed in *me*/+ mice.

Body

As we described in the annual report provided last year, we encountered a number of unexpected difficulties in animal husbandry due to problems in the animal facility, which were beyond our control. These circumstances had shifted the focus of our studies on characterizing the newly generated transgenic mice that are the basis of aim 2 of our original application.

The original hypothesis of aim 2 was to test whether the increased incidence of breast cancer in *me*/+ mice is specifically due to impaired SHP-1 function in the T cell lineage. In order to address this question, we have generated mice that carry dominant negative mutants of SHP-1 (C453S and D419A) using the Cre/loxP system sites (2, 3) that allows tissue-specific expression upon crossing into Cre-expressing mouse strains (Fig. 1). We have obtained 2 (C453S) and 4 (D419A) independent transgenic lines that are currently further characterized.

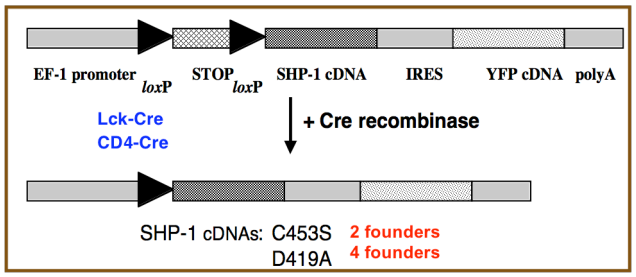

Figure 1: Transgenic construct to generate mice expressing wild type and dominant negative mutants of SHP-1 (C453S and D419A) in a tissue-specific manner.

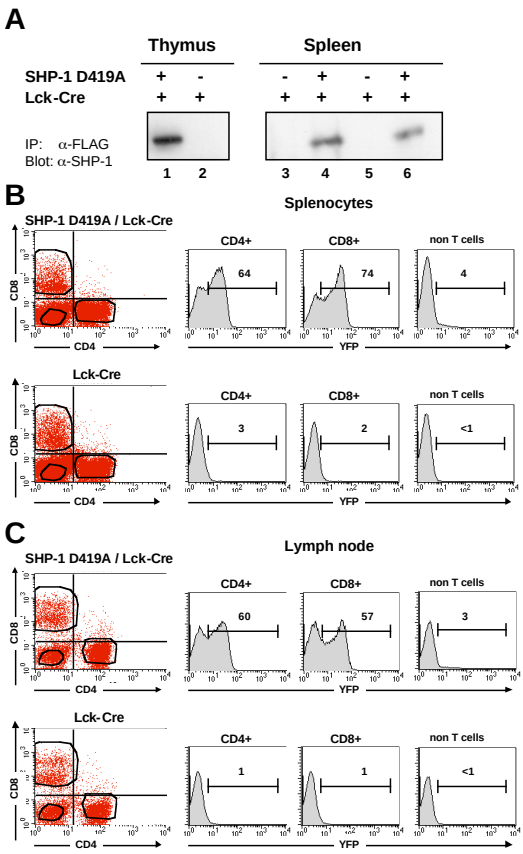

Figure 2: *Mice double transgenic for SHP-1 D419A and Lck-Cre express transgene.* (A) Transgenic flag-tagged SHP-1 D419A was immunoprecipitated from lysates of thymi (2 x 10^7 cells) or spleens (lanes 3 and 4: 4 x 10^6 and lanes 5 and 6: 10^6 cells/IP) of mice carrying indicated genotypes followed by immunoblot with anti-SHP-1. Flow cytometric profiles shown in B and C are from mice analyzed in lanes 5 and 6. Spleens (B) and lymph nodes (C) were isolated from 8 week old mice either transgenic for SHP-1 D419A and Lck-Cre (top of A and B) or for Lck-Cre alone (bottom of A and B). Cellular suspensions were stained for flow cytometry with the indicated antibodies. Gated subpopulations were analyzed for YFP expression. Cells not positive for CD4 or CD8 are referred to as non T cells. Percentages of YFP+ cells within each subpopulation are indicated.

Upon crossing with Lck-Cre mice, expression of the SHP-1 mutants is targeted to the T cell lineage. Analysis of the first generations of mice showed lineage-specific expression as assessed by western blotting and flow cytometry (Fig. 2).

To evaluate whether the putative dominant negative mutants for their effect on TCR-mediated signaling, we assessed TCR/CD3-driven proliferation in mice expressing dominant negative SHP-1 in the T cell lineage. In particular, TCR-induced proliferation of splenic T cells derived from SHP-1 D419A and *lck*-Cre double transgenic mice or the control mice single transgenic for either SHP-1 D419A or *lck*-Cre. T cells derived from double transgenic 12-14-week old mice were hypersensitive to TCR stimulation (Fig. 3). Interestingly, the 2-3 fold leftward shift observed in mice expressing the dominant negative SHP-1 mutant compared to controls is highly similar to the phenotype observed in SHP-1-deficient *me/me* T cells (4, 5). We are very encouraged by this finding, since it further validates that T cell-specific expression of the SHP-1 D419A mutant mimicks the *me/me* T cell phenotype. Moreover, since only the T cell lineage was affected by the loss of SHP-1 in

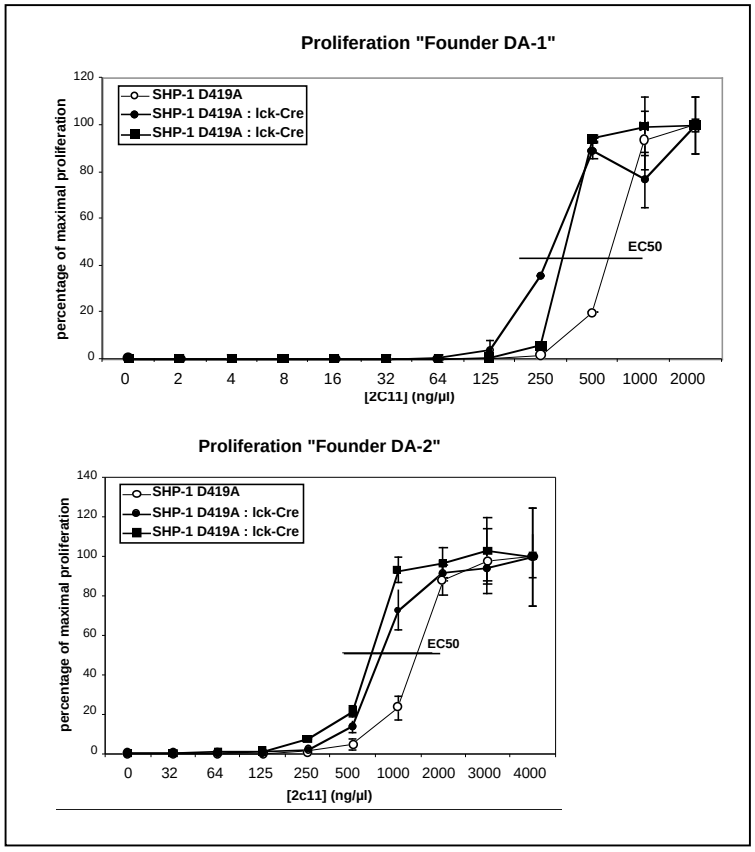

Figure 3: *T cells from transgenic mice expressing dominant negative mutant of SHP-1 are hyper-sensitive to TCR/CD3 stimulation.* Proliferative response of T cells derived from single (SHP-1 D419A) or double (SHP-1 D419A : *lck*-Cre) transgenic mice in response to increasing amounts of anti-CD3ε antibody (2c11). Splenocytes were enriched for T cells using negative selection via magnetic beads (Miltenyi). 5 x 10^4 T cells were stimulated with the indicated concentrations of plate-bound anti-CD3ε. After 72 hrs., cells were pulsed with 1 µCi of [^{3}H] Thymidine for 18 hrs. [^{3}H] Thymidine incorporation was measured using a cell harvester. The data are presented as percentage of maximal incorporated counts at the highest concentration of anti-CD3ε. In general maximal incorporation was ~200.000 cpm. The two graphs represent experiments using offspring (~3 month old) from two different founder mice (DA-1 and DA-2). Error bars represent the standard deviation of the mean.

these studies, rather than the entire hematopoietic system as in *me/me* mice, it suggests that the negative regulatory role of SHP-1 is a T cell-autonomous effect.

genotype	**N**	**CD4 T cells (%)**	**CD4+CD25+** (% within CD4+ population)	**p value**
Lymph node				
SHP-1 D419A**-** / Lck-Cre**+**	**3** (4)	**47.0±4.3** (44.9±5.6)	**5.0±0.9** (5.7 ± 1.8)	**0.0057** (0.1364)
SHP-1 D419A**+** / Lck-Cre**+**	**4**	**42.2±4.5**	**7.0 ±0.2**	
Spleen				
SHP-1 D419A**-** / Lck-Cre**+**	**3** (4)	**19.3±0.5** (18.2±2.6)	**6.9±1.3** (7.7 ±2.1)	**0.0174** (0.0949)
SHP-1 D419A**+** / Lck-Cre**+**	**4**	**13.7 ±1.9**	**9.9±1.0**	

Table 1: CD4+CD25+ Treg cell analyses of D419A expressing and non-expressing mice. N=number of mice analyzed in each group. Initially 4 mice were analyzed in each group. However upon further analysis, one outlier was identified in the SHP-1 D419A- / Lck-Cre+ group and based on the Grubb's test for outliers, this data point was omitted for the final analysis. Data including this outlier are provided in parentheses. p values were calculated for the changes in percentages of CD4+CD25+ Treg cells within the CD4 T cell population. Statistical analyses showed a statistically significant increase in the percentage of Treg cells both in the lymph nodes as well as in the spleen of mice expressing the SHP-1 D419A mutant compared to non-expressing mice.

We then assessed whether expression of the D419A mutant solely in the T cell lineage affects the number of CD4+CD25+ Treg cells in these transgenic mice. A preliminary analysis of spleen and lymph nodes of a limited number of mice showed a promising statistically significant increase in the number of CD4+CD25+ Treg cells in mice expressing the D419A mutant compared to non-expressers (in thymus ~7% vs. 5%, in spleen and lymph nodes ~10% vs. 7%) (Table 1). We realize that these are preliminary relatively small changes; however, the observed increase in CD4+CD25+ Treg cells is comparable to our previous data obtained from *me/me* mice (1), which further supports the feasibility of the proposed studies to test the hypothesis that increased numbers of Treg cells contribute to the development of breast cancer using these transgenic mice.

However, while we were performing these studies and were in the process of setting up additional backcrosses, we noticed that our mice carrying the Lck-cre gene started to show deletion of the loxP sites even in non-T cell tissues. For example, tail biopsies showed almost 100% deletion. Interestingly, at the same time, we became aware that Taconic where we had originally purchased our mice had posted the following statement at their website (http://www.taconic.com/wmspage.cfm?parm1=800): "*Dr. Chris Wilson (cbwilson@u.washington.edu) has recently discovered that his TgN(Lck-Cre) mouse can cause partial to complete deletion in non-lymphoid tissues in some mice on certain backgrounds. These occurrences are unpredictable, but are apparent in tail DNA samples. Investigators should determine whether this abnormality is present in their crosses.*" We therefore switched from Lck-Cre to CD4-Cre where such aberrant deletion has not been observed. CD4-Cre had also originally been developed by Dr. Chris Wilson. It uses the mouse CD4 promoter/enhancer/silencer, which first allows expression of CD4-driven Cre as thymocytes enter the double positive stage. We have now crossed the SHP-1 mutant transgenic mice with CD4-Cre and found reliable T cell-specific deletion of the loxP sites in these mice. Initial analyses confirmed that T cells expressing a dominant negative mutant of SHP-1 were hyper-responsive to TCR-mediated stimulation. We have performed these experiments in response to unspecific anti-CD3 crosslinking as well as in response to cognate peptide (OVA) in mice that were crossed to transgenic TCR (OT-II)-expressing mice. While we observe consistent

expression of the SHP-1 mutant transgene in mice that co-express CD4-Cre, we still see wide variations in expression levels. We are currently trying to generate lines with more consistent expression levels by backcrossing mice that consistently produce litters with higher expression levels.

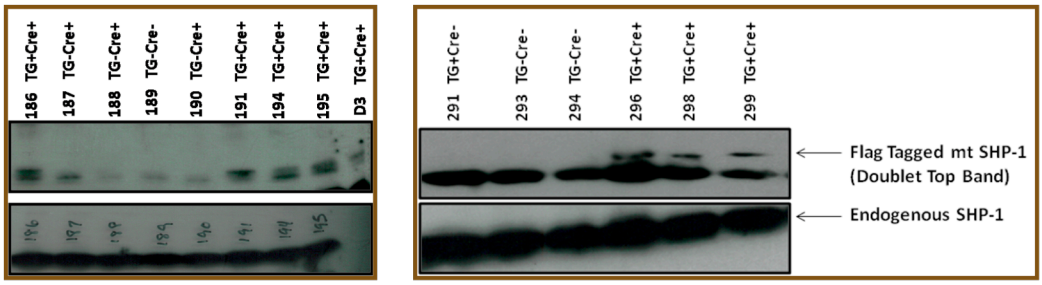

Figure 4: *Mice double transgenic for SHP-1 D419A and Lck-Cre express transgene to varying degress.* Transgenic flag-tagged SHP-1 D419A and endogenous SHP-1 were immunoprecipitated from thymic lysates (1 x 10^7 cells/point) using mouse monoclonal anti-Flag antibodies and rabbit polyclonal anti-SHP-1 serum respectively followed by immunoblot with anti-SHP-1. Genotypes of individual samples are indicated.

Because of the inconsistent expression levels (Fig. 4), we have no yet obtained statistically significant data with respect to the number of Treg cells in mice of these new crosses. At this point, we have not observed any incidence of breast cancer in these mice. However, the majority of mice are still much younger (< 6 mo) than the average age of me/+ mice with mammary tumors (~ 12-15 mo). We are continuing to monitor these mice for tumor development over the next months.

Key Research Accomplishments

- Generation of transgenic mice expressing the SHP-1 D419A mutant specifically in the T cell lineage through the use of Lck-Cre
- D419A expressers show T cell autonomous effect of SHP-1 as assessed by TCR/CD3-mediated hyper-proliferation
- D419A expressers show increased numbers of CD4+CD25+ Treg cells
- Re-generation of transgenic mice expressing the SHP-1 D419A mutant specifically in the T cell lineage CD4-Cre transgenic mice

Reportable Outcomes

Poster presented at Era of Hope meeting in Baltimore (June 2008)
Ulrike Lorenz, Tessy Iype, Mohan Sankarshanan, Ileana Soto, and Gina Calabrese
"*Role of the Tyrosine Phosphatase SHP-1 and Regulatory T cells in Breast Cancer"*

Conclusion

Based on the preliminary data obtained from the SHP-1 D419A/Lck-Cre mice, which demonstrate a T cell autonomous effect of SHP-1 as evidenced by effects on T cell proliferation and increased CD4+CD25+ Treg numbers, we conclude that the approach proposed in aim 2 (analysis of mice deficient in SHP-1activity in the T cell lineage) of our original application is feasible and is in fact better suited than the alternative approach of the original aim 1 (analysis of mice deficient in SHP-1activity in all SHP-1-expressign lineages) to directly address the hypothesis that SHP-1 deficiency promotes the development/onset of breast cancer by increasing the number of regulatory T cells. However, due to the unexpected onset of non-tissue-specific deletion by the Lck-Cre, we recently switched to CD4-Cre. Our initial analyses of these mice confirmed the T cell autonomous effect of SHP-1 with respect to T cell proliferation. However, we have no yet statistically significant data with respect to the number of Treg cells in mice of these new crosses. At this point, we have not observed any incidence of breast cancer in these mice. However, the majority of mice are still much younger (< 6 mo) than the average age of me/+ mice with mammary tumors (~ 12-15 mo). We will continue to monitor these mice for tumor development over the next months.

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Appendices

Not applicable

Personnel Involved in the Research Effort

- Ulrike Lorenz (Principal Investigator)
- Gina Calabrese (Laboratory Sepcialist Senior)
- Tessy Iype (Research Associate)